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14. ABSTRACT

The main objective of this research project is to test the hypothesis that a newly discovered retrovirus, XMRV, is involved development of human breast cancer. To accomplish this, XMRV protein and nucleic acid will be detected in breast cancer specimens that have been formalin-fixed and paraffin-embedded (FF-PE). We have obtained the required number of FF-PE breast specimens for this study from Asterand, Inc., Detroit, MI. We have optimized assay conditions for the detection of XMRV protein PE sections by the identification of an antibody that is at least four-fold more sensitive than other antibodies that have been previous studies. This was done by our production of cell lines that express infectious XMRV virus by starting with plasmid containing the cloned XMRV genome. With this antibody we have been able to detect XMRV protein by immunohistochemistry analysis of these cell lines. These results will thus enable us to carry out the IHC analysis of the FF-PE breast cancer specimens.

15. SUBJECT TERMS
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INTRODUCTION: A novel retrovirus, xenotropic murine leukemia virus-related virus (XMRV), has recently been linked to prostate cancer (1-2). XMRV was detectable in 23% of prostate cancer patients (2) and 4% of healthy controls (3). XMRV infection, moreover, correlated predominantly with high-grade prostate cancers (2). To determine whether XMRV is involved in the pathogenesis of other types of human cancer, such as breast cancer, we have proposed to examine breast cancer specimens for the presence of XMRV protein and nucleic acid. Breast cancer tissue that has been formalin-fixed and paraffin-embedded will be subjected to immunohistochemistry analysis using XMRV-specific antibodies. As an independent assay for XMRV detection, viral nucleic acid will be assessed by either fluorescence *in situ* hybridization or RT-PCR. Our statistical analysis has indicated that the detection of XMRV-positive cancers at a rate of 16% or greater will require 28 patient samples, assuming a 4% incidence rate in the general population (3), alpha = 0.05 (1-sided) and power = 0.80. A demonstration that an infectious retrovirus is involved in the development of breast cancer could potentially lead to the development of a new biomarker for detection, diagnosis, and prognosis. It would further provide new forms of treatment and ultimately prevention of this deadly disease, which is the leading cause of cancer death among Hispanic women and second for women of all other races.

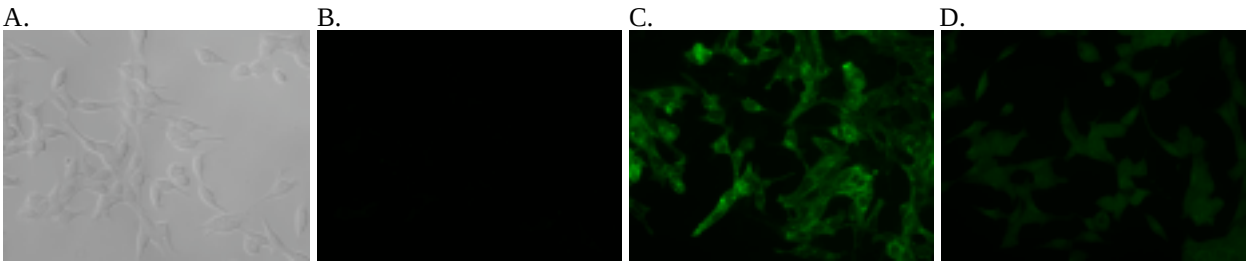
BODY: To determine whether XMRV is associated with human breast cancer, we have proposed to examine formalin-fixed and paraffin-embedded (FF-PE) breast cancer tissue for viral protein and nucleic acid by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH), respectively. We have initiated these studies by generating XMRV-infected cultured cells for use as essential controls for these assays. The production of virus-infected cells first required the preparation of infectious virus. This was accomplished by transfecting human 293T cells with plasmid DNA containing a clone of the XMRV genome, which was obtained from Dr. Robert Silverman (The Cleveland Clinic, 4). Transfection was performed by treating cells with Lipofectamine 2000 (Invitrogen). Cell-free supernatant was collected and used to infect cultured human LNCaP cells. These cells were chosen because they are highly susceptible to XMRV infection (2).

To detect XMRV-infected LNCaP cells, we used an indirect immunofluorescence assay that we have used in our previous studies of other types of murine leukemia viruses (MLVs) (5). For this assay, we used a rat monoclonal antibody (MAb) 83A25, which recognizes the envelope glycoprotein of different MLVs, some of which are closely related to XMRV (5, 6), and obtained successful XMRV detection (Fig. 1C). We subsequently compared the sensitivity of this antibody with a rat anti-SFFV MAb that recognizes the XMRV Gag protein and has been used to detect XMRV in previous studies by other laboratories (1). Our results showed that the 83A25 MAb was at least 4-fold more sensitive than the anti-SFFV MAb (Fig. 1). Briefly, this assay involved the growth of uninfected or XMRV-infected LNCaP cells on an 8-well chamber slide coated with poly-L-lysine, followed by incubation with either 83A25 or anti-SFFV MAb. After rinsing, cells were treated with a goat anti-rat fluorescent antibody (Alexa Fluor 488, Invitrogen). Fluorescence quantification was performed with a Zeiss Axioplan 2 microscope and Metamorph software provided by the Karmanos Cancer Institute Microscopy, Imaging and

Cytometry Resources (KCI MICR) Core facility. Based on our results, we have chosen to use the 83A25 MAb for IHC analysis.

As essential controls for IHC and FISH assays of FF-PE breast cancer tissues, we will use FF-PE sections containing uninfected or XMRV-infected LNCaP cells. For this purpose, FF-PE sections of these cultured cells have been prepared by following standard procedures (7). To initiate IHC studies, we have optimized conditions for these assays using the 83A25 MAb as the primary antibody. 5-mm sections were cut and placed on electrically-charged glass slides and dried at 56°C for 30 min. Sections were deparaffinized in xylene and rehydrated in decreasing alcohol concentrations (100%, 95%, 85%, 3 min each). Antigen retrieval was performed via steam treatment for 20 min in 1mM EDTA. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. Sections were incubated with 83A25 hybridoma supernatant overnight at 4°C. After washing with PBS 3x at room temperature (RT), sections were incubated with a secondary anti-rat biotinylated antibody (Ventana Medical Systems) for 30 min at RT, and treated with a streptavidin-horseradish peroxidase conjugate (ABC) reagent. Viral protein was detected with a 3-amino-9-ethylcarbazole (AEC) chromogen reaction. Sections were counterstained with hematoxylin for 2 min at RT and dehydrated with graded alcohols. Slides were sealed with Cytoseal 60 mounting media (Thermo Scientific). FF-PE uninfected and XMRV-infected LNCaP cells that were analyzed by IHC are shown in Fig. 2. These optimized conditions will be used for IHC analysis of FF-PE breast cancer tissues.

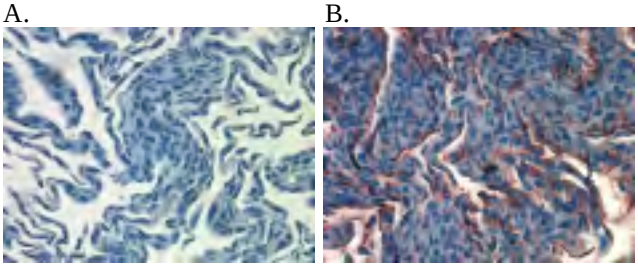
Fig. 1. Identification of a monoclonal antibody for the detection of XMRV infection.



Comparison between 83A25 and anti-SFFV MAbs for the detection of XMRV protein by indirect immunofluorescence. (A) Bright field microscopy of XMRV-infected LNCaP cells at 200x magnification using a Zeiss Axioplan 2 fluorescence microscope (KCI MICR Core). Fluorescence images of XMRV-infected LNCaP cells treated with either (B) secondary goat anti-rat fluorescent (Alexa Fluor 488) Ab and no primary MAb; (C) primary 83A25 MAb and secondary fluorescent Ab; or (D) primary anti-SFFV MAb and secondary fluorescent Ab.

Fig. 2. Detection of XMRV infection of LNCaP cells by IHC analysis.

A. B.



(A) Uninfected and (B) XMRV-infected LNCaP cells were formalin-fixed and paraffin-embedded. 5-mm sections were cut and subjected to IHC analysis with 83A25 MAb and the Ventana BASIC AEC Detection kit for horseradish peroxidase activity. Sections were counterstained with hematoxylin revealing blue nuclei. Photomicrographs were taken at 400x magnification with a Zeiss Axiophot microscope (KCI MICR Core).

KEY RESEARCH ACCOMPLISHMENTS:

- Infectious XMRV was prepared by transfecting human 293T cells with plasmid DNA containing a clone of the XMRV genome (obtained from Robert Silverman, The Cleveland Clinic, 4). Cell-free supernatant containing infectious virus was collected.
- XMRV-infected LNCaP cells were detected with an indirect immunofluorescence assay with the 83A25 monoclonal antibody (MAb) that recognizes the glycoprotein of various types of xenotropic murine leukemia virus (5-6).
- XMRV-infected LNCaP cells were used to compare the 83A25 MAb with the anti-SFFV MAb for virus detection by indirect immunofluorescence (Fig. 1). The anti-SFFV MAb was chosen for comparison because it was used for XMRV detection in previous studies (1). Quantitative immunofluorescence analysis with a Zeiss Axioplan 2 microscope and Metamorph software indicated that the 83A25 MAb had at least four-fold greater sensitivity for virus detection in comparison with the anti-SFFV MAb.
- XMRV-infected and uninfected LNCaP cells were formalin-fixed and paraffin-embedded as positive and negative controls for immunohistochemistry (IHC) analysis. 5mm-sections were cut for the optimization of IHC conditions with the 83A25 MAb (Fig. 2). FF-PE sections of these cells will be used as controls for IHC analysis of FF-PE breast cancer tissues.
- 28 de-identified breast cancer FF-PE tissue samples have been obtained from Asterand, Inc., Detroit, MI, for IHC analysis using our optimized protocol. These tissue samples will also be used for the detection of XMRV nucleic acid by fluorescence *in situ* hybridization.

REPORTABLE OUTCOMES: A XMRV-infected LNCaP cell line has been produced, and a monoclonal antibody has been identified for sensitive XMRV-detection by IHC. Optimum conditions for IHC detection of FF-PE XMRV-infected LNCaP cells have been developed.

CONCLUSION: We have developed cell lines for detection of XMRV infection by immunohistochemistry and identified a monoclonal antibody that detects this retrovirus with greater sensitivity than that used in previous studies by other laboratories. Using this monoclonal antibody and cell lines as controls, we have optimized conditions for performing IHC analysis of FF-PE breast cancer tissues. These conditions will be used for the analysis of the breast cancer specimens we have obtained from a commercial source. The results of the IHC analysis and that of XMRV nucleic acid will enable us to determine whether there is an association of XMRV infection with breast cancer. The involvement of an infectious retrovirus in the development of breast cancer could provide a new biomarker for diagnosis and prognosis, especially, if similar to prostate cancer, viral presence especially correlates with tumors of high-grade (2). Effective treatment would be achieved by the use of antiretroviral drugs that are already in extensive and effective use to treat patients infected with HIV, another retrovirus. The development of a vaccine against infection by XMRV could ultimately result in the prevention of this deadly disease.

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